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Soluble inflammatory mediators induce transcriptional re-organization that is independent of dna methylation changes in cultured human chorionic villous trophoblasts

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ABSTRACT

The studies proposed here were undertaken to test the hypothesis that, under specific circumstances (e.g., a strong enough inflammatory stimulus), genes that are repressed at the maternal-fetal interface via DNA methylation might be de-methylated, allowing either a maternal immune response to the semi-allogenic fetus or the onset of early labor. Chorionic trophoblasts (CT) were isolated from fetal membranes, followed by incubation with medium from LPS-activated PBMC or resting PBMC medium for 2 h. RNA and DNA were isolated from the cells for RNA-seq and DNA methylation studies. Two hrs after being exposed to conditioned medium from LPS-activated PBMC, CT showed differential expression of 114 genes, all but 2 of which showed higher expression in the stimulated cells than is the unstimulated cells. We also identified 318 differentially methylated regions (DMRs) that associated with 306 genes (155 protein coding genes) in the two groups, but the observed methylation changes had negligible impact on the observed transcriptional changes in CT.

CT display complex patterns of transcription in response to inflammation. DNA methylation does not appear to be an important regulator of the observed transcriptional changes.

1. Introduction

Preterm labor and premature birth remain a significant cause of neonatal mortality and morbidity. Numerous investigations have demonstrated that multiple risk factors are associated with preterm labor. These include genetic variance (Zhang et al., 2015), pregnancy complications such as pre-eclampsia (Schuiling et al., 1997; Redman and Sargent, 2005; Visser et al., 2007), and intra-uterine infection or inflammation (Terzidou and Bennett, 2002; Romero et al., 2007). In addition to the risk for preterm labor, inflammation *in utero* has also been linked to neonatal complications, including both respiratory and neurologic morbidities (Yoon et al., 1997; Gomez et al., 1998; Edwards and Tan, 2006; Kallapur and Jobe, 2006). Furthermore, there is growing evidence that fetal morbidities may be mediated through specific responses of cells within the placenta and/or fetal membranes (Pappas et al., 2015).

Successful pregnancy requires significant alterations in maternal immune responses, balancing factors that permit survival of the semi-

allogenic fetus with the need for sufficient immune function to maintain the mother's ability to protect herself and her fetus from invading micro-organisms (Jarvis et al., 2004; Guleria and Sayegh, 2007). Furthermore, specific immunologic functions must be intact to properly regulate implantation and placentation (Loke and King, 2000; Abrahams and Mor, 2005; Knofler et al., 2005; van den Brule et al., 2005; Bao et al., 2006). The trophoblast, the most abundant cell at the maternal-fetal interface, is immunologically active and participates in both the early events establishing pregnancy and in maintaining immunologic balance as pregnancy ensues (Guleria and Pollard, 2000; Hauguel-de Mouzon and Guerre-Millo, 2006; Jiang et al., 2006). The ability of trophoblasts to maintain and restore immunological homeostasis is suggested by work in trophoblast cell lines, where perturbation of gene expression patterns by inflammation is restored within 6 h of the initial stimulus (Jarvis et al., 2004).

While there has been considerable interest in transcriptional regulation in the maintenance of immune homeostasis in the placenta, considerably less is known about the fetal membranes. These

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membranes consist of the amnion (the innermost layer) and the chorion, which is connected to the maternal decidua and plays a critical role in maintaining maternal-fetal immune tolerance. The chorionic layer is rich with trophoblasts that synthesize multiple important hormones (for example (Sun et al., 1998)), and failure of these cells to grow and function normally as been associated with poor fetal growth (Murthi et al., 2005). Chorionic trophoblasts (CT) provide an important line of defense against infection, and chorioamnionitis is an important trigger for pre-term labor (Vrachnis et al., 2010). The latter observation is consistent with our hypothesis that, while cells at the maternal-fetal interface are particularly effective in maintaining the delicate immune/hormonal state that is essential for mammalian pregnancy (Jarvis et al., 2004), these mechanisms can be overwhelmed (this capacity of cells to maintain homeostasis despite even extreme perturbations in the external or internal environments is sometimes called *robustness* and can be observed, characterized, and measured at the genome level (Macneil and Walhout, 2011)). Thus, an important task before the field of reproductive immunology is to understand the mechanisms that regulate gene expression in relevant cells at the maternal-fetal interface, and in particular the mechanisms through which cellular/tissue homeostasis is maintained in the setting of environmental challenges, including exposure to toxins and infectious agents. Since the epigenome is an important means through which cells sense and respond to environmental cues (Jirtle and Skinner, 2007), such studies will invariably require an understanding of the epigenetic features of relevant cells that regulate gene expression.

The studies described here were undertaken to test the hypothesis that, under specific circumstances (e.g., a strong enough inflammatory stimulus), genes that are repressed at the maternal-fetal interface via DNA methylation might be de-methylated, allowing either a maternal immune response to the semi-allogenic fetus or the onset of early labor. We report that, while chorionic trophoblasts display specific transcriptional changes and in an *in vitro* model of inflammation, these changes are not driven by changes in the DNA methylome.

2. Materials and methods

2.1. Preparation of conditioned medium to stimulate CT

Whole blood was obtained by venipuncture from two healthy, non-pregnant women under the age of 40 years. Peripheral blood mononuclear cells (PBMC) were prepared by density gradient centrifugation and stimulated with bacterial lipopolysaccharide (LPS) exactly as described in (Jiang et al., 2007). PBMC were cultured for 24 h with or without LPS, after which medium was subjected to centrifugation to remove cellular debris and frozen at -80°C until used to stimulate CT. We have previously reported that conditioned medium produced in this fashion produces cytokine levels that display little inter-donor variability: IL1b: 2.54 ± 0.53 ng/ml, TNFa: 2.32 ± 0.35 ng/ml, IFNg: 0.23 ± 0.05 ng/ml, IL6: 16.92 ± 3.47 ng/ml (Jiang et al., 2006). The protocol for obtaining blood from healthy young adults to prepare conditioned medium was reviewed and approved by the University at Buffalo IRB. All research was carried out following the protocol as reviewed and approved.

2.2. Preparation of chorionic trophoblasts (CT) and cell stimulation

Whole placentas were obtained from healthy women between ages 18–40 who were undergoing routine repeat C-sections. Samples were excluded if the mother reported fever in the 36 h prior to delivery or had a documented vaginal infection during the course of pregnancy. The protocol for the acquisition of human placentas for the preparation of chorionic villous trophoblasts was reviewed by the University of Buffalo Institutional Review Board (IRB) and was determined to not fall under the category of human subjects research.

Chorionic trophoblasts were prepared following Li's protocol (Li

et al., 2006). Briefly, chorion was peeled off the amnion, and the residual blood was washed off chorionic tissue with cold normal saline. The minced chorionic tissue was digested with 0.125% (wt/vol) trypsin (Life Technologies, Inc., Grand Island, NY) and 0.1% (wt/vol) collagenase type 1 (Life Technologies) three times for 60 min. The chorionic cells were loaded onto a stepwise 5–75% (vol/vol) Percoll (Fisher Scientific, Hanover Park, IL) gradient composed of increments of 5% Percoll and centrifuged at $2500 \times g$ for 20 min to separate different cell types. Cytotrophoblasts between the density markers of 1.049 and 1.062 g/ml (corresponding to 35 and 50% Percoll) were collected and plated at a density of 5×10^6 cells per T25 flask in DMEM culture medium (Life Technologies) containing 10% (vol/vol) fetal calf serum (FCS) (Life Technologies) and antibiotic-antimycotic (Life Technologies). Cells were cultured at 37°C in 5% CO_2 in air. Cells prepared using the above method are predominantly chorion-derived trophoblast rather than decidual cells (Sun et al., 1997). Cells were culture for 3 days. Cells were then stimulated with LPS-activated PBMC medium (LPS sample) or resting PBMC medium (control sample) for 2 h. This time frame was derived empirically from our studies in human choriocarcinoma cells, in which we observed maximal transcriptional responses to inflammatory stimuli at 2 h, with almost complete restoration by 8 h (Jarvis et al., 2004). Half of the samples were stimulated by conditioned medium from one healthy donor, and half were stimulated with conditioned medium from the other. Cells were then collected and lysed in Trizol or store at -80°C until used for DNA methylation study.

2.3. RNA isolation and sequencing

Total RNA was extracted from Trizol[®] reagent according to the manufacturer's directions, including a DNase (Qiagen) step to remove genomic DNA. RNA was quantified spectrophotometrically (Nanodrop, Thermo Scientific, Wilmington, DE) and assessed for quality by capillary gel electrophoresis (Agilent 2100 Bioanalyzer; Agilent Technologies, Inc., Palo Alto, CA). cDNA libraries were prepared for each sample using the Illumina TruSeq RNA Sample Preparation Kit by following the manufacture's recommended procedures. Libraries were sequenced using 50 base pair (bp) single-end reads on Illumina HiSeq 2500 instruments. Library construction and RNASeq were performed at the University at Buffalo Genomics and Bioinformatics Core.

2.4. Differential gene expression analysis

Illumina sequencing paired-end reads were aligned to the human reference genome hg19 downloaded from the University of California Santa Cruz Genome Bioinformatics Site, UCSC using TopHat (Trapnell et al., 2009). Mapping analysis resulted an average of 129 million mapped reads per sample, average mapping rate of 86% over all samples (Supplementary Table S1).

We computed gene expression abundance using the htseq-count function from the HTSeq Python package (Anders et al., 2015) with reference to GENCODE v19 gene annotation (Harrow et al., 2012). Gene expression counts were then used for differentially expressed (DE) genes analysis using limma R package. DEGs were defined as those genes with minimum fold change (FC) of 1.5 and adjusted p-value ≤ 0.05 .

2.5. GO analysis of differentially expressed genes

Gene ontology (GO) enrichment analysis of DE genes was performed using Gene Ontology enRichment analysis and visualiZation tool (GORilla) (Eden et al., 2009) to assess the biological processes associate with DE genes. REduce Visualize gene ontology (REVigo) was used to classify GO terms base on semantic similarity measurement (Supek et al., 2011).

2.6. Corroboration of differential expression by quantitative real time RT-PCR (qRT-PCR)

An independent cohort of 4 LPS samples and 4 control samples was used for validation using qRT-PCR. Total RNA was reverse transcribed with iScript™ cDNA synthesis kit according to the directions of the manufacturer (Bio-Rad, Hercules, CA, USA). Real-time RT-PCR was performed using SYBR Green reagents on a StepOne Plus (Applied Biosystems, Foster City, CA, USA) as described previously (Jiang et al., 2015). Gene-specific amplification was confirmed by a single peak in the ABI Dissociation Curve software. The relative abundance of transcript expression data was normalized to GAPDH expression. Results are presented as the ratio of the concentration of messenger RNA (mRNA) relative to GAPDH mRNA ($2^{-\Delta Ct}$). The nucleotide sequences of the primers were as follows. Statistical analysis was performed on the ΔCt value using unpaired *t*-tests. Primers were synthesized by Integrated DNA Technologies. The nucleotide sequences of the primers were as follows: CCL4, 5′-CCTCGCAACTTTGTGGTAGA-3′ (Forward) and 5′-GTTCCAGTTCATACACGTACTC-3′ (Reverse); CXCL2, 5′-TCACC TCAAGAACATCCAAAGT-3′ (Forward) and 5′-CAAGCTTTCTGCCCATTTCTTG-3′ (Reverse); ICAM1, 5′-ATGGGCAGTCAACAGCTAAA-3′ (Forward) and 5′-CAGCGTAGGGTAAGGTTCTTG-3′ (Reverse); IER3, 5′-ATCTTCACCTTCGACCTCT-3′ (Forward) and 5′-ACCACTCGAGGG TAGAGAAC-3′ (Reverse); IL1B, 5′-ATGGACAAGCTGAGGAAGATG-3′ (Forward) and 5′-CCCATGTGTGGAAGAAGATAGG-3′ (Reverse); IL6, 5′-CACTCACCTCTTCAGAACGAAT-3′ (Forward) and 5′-GCTGCTTCA CACATGTTACTC-3′ (Reverse); LIF, 5′-AGCCCATAAATGAAGGTCT TGG-3′ (Forward) and 5′-TGAGGTTGTGTGACATGGG-3′ (Reverse); NFKB1A, 5′-AGAGAGGATTTCCGTTTCCGTTAT-3′ (Forward) and 5′-CTTTGCTGGTCCCACATAGT-3′ (Reverse); PLA2, 5′-CCAAAGAAG GAGGACTACATCG-3′ (Forward) and 5′-CTCCTTGAACGGATCTT CAG-3′ (Reverse); SDC4, 5′-GAAGTTGTCCATCCCTTGGT-3′ (Forward) and 5′-CTCCTCTAGTTTCTTGGGTTCG-3′ (Reverse). Primers were synthesized by Integrated DNA Technologies.

2.7. Upstream analysis using Ingenuity software

To identify upstream regulators of the differentially expressed genes between two groups, we used IPA software (Ingenuity Systems, Redwood City, CA). Gene symbols were used as identifiers and the Ingenuity Knowledge Base gene set as a reference for a pathway analysis. Identification of upstream transcription regulator was assessed using IPA where the activation or inhibition of a transcription regulator was determined from expression patterns of the transcription factor and its downstream-regulated genes within the differentially expressed list. The absolute value of the *z*-score ≥ 2.0 was considered statistically significant with a positive value indicating activation and a negative values indicating inhibition of the transcription factor.

2.8. Genome-wide assessment of DNA methylation

MeDIP and MRE sequencing libraries were generated as described previously (Li et al., 2015) with minor modifications. This approach allowed us to directly assess both the methylated (MeDIPseq) and unmethylated (MREseq) portions of the genome. Thus, the findings from each sequencing approach corroborate the other on a genome-wide scale, obviating the need to independent corroboration of selected sites using bisulfite conversion-sequencing.

Briefly, for MeDIP-seq, 500 ng of DNA isolated was sonicated, end-processed, and ligated to paired-end adapters. After agarose gel size-selection, DNA was immunoprecipitated using a mouse monoclonal anti-methylcytidine antibody. Immunoprecipitated DNA was washed, amplified by 12 cycles of PCR, and size-selected by agarose gel electrophoresis. For MRE-seq, 5 parallel digests (HpaII, Hin6I, SsiI, BstUI, and HpyCH4IV; New England Biolabs) were performed, each with 200 ng of DNA. Digested DNA was combined, size-selected, end-

processed, and ligated to single-end adapters. After the second size-selection, DNA was amplified by 15 cycles of PCR and size-selected by agarose gel electrophoresis.

MeDIP and MRE libraries were sequenced on Illumina HiSeq machine with an average of number of approximately 1.77 billion MeDIP-seq reads and 714 million MRE-seq reads. These reads were aligned using BWA (Li and Durbin, 2009) against human genome reference hg19, an average of 86% and 85% mapping rates of for MeDIP-seq and MRE-seq respectively (Supplementary Tables S2 and S3).

To identify methylation regions in placenta trophoblast, we first performed genome wide CpG density analysis on MeDIP-seq and MRE-seq aligned reads for every sample independently using methylQA R package (Li et al., 2015). For each sample, the output file in bed format obtained from CpG density analysis was used as input for computing number of uniquely mapped reads in non-overlapping 500 basepairs (bp) window across the entire genome. Uniquely mapped reads were then transformed into read per kilo bases per million reads (RPKM) format. We denoted methylation site (500 bp) as region with average MeDIP-seq RPKM ≥ 1 across all samples and average MRE-seq RPKM < 1 .

2.9. Identification of differentially methylated regions

We used methylMnM R package (Zhang et al., 2013) to detect differentially methylated regions (DMR) between LPS sample and Control sample, significant DMR as those with adjusted *p*-value < 0.05 and minimum fold change of 1.5.

2.10. Assessment of the influence of methylation changes on transcription

We sought to assess whether methylation changes in response to LPS activated PBMC medium exposure are correlated with transcriptome alteration. We used BETA program (Wang et al., 2013) to search for methylation changes (DMR) that contribute to transcriptional activation or suppression.

3. Results

3.1. Differential gene transcription in response to an inflammatory stimulus

We used an *in vitro* model to examine transcriptional responses in CT (derived from uncomplicated term pregnancies) to soluble mediators from LPS-stimulated PBMC. We compared findings from CT incubated with conditioned medium from unstimulated PBMC. Prior to differential gene expression analysis, we performed principal component analysis over gene expression values from the CT to preview the distribution of samples in two the groups (LPS samples or control samples). We identified three LPS samples that clustered closely with control samples (Supplementary Fig. 1). We excluded these three paired samples in order to obtain a clearer view of genes that showed unambiguous differential expression between the two sample sets. Two hr after being exposed to conditioned medium from LPS-activated PBMC, CT showed differential expression of 114 genes. All but 2 of these genes showed higher expression in the cells incubated with conditioned medium from LPS-stimulated PBMC (“stimulated cells”) than in the cells incubated with conditioned medium from unstimulated PBMC (“unstimulated cells” - Supplementary Table 4). These results paralleled what we have previously seen in choriocarcinoma cell lines (Jarvis et al., 2004). Hierarchical cluster analysis (Fig. 1) crisply separated the stimulated and unstimulated cells. Additional analysis did not reveal clustering based on whether cells had been stimulated by one conditioned medium sample vs the other.

Gene ontology (GO) analysis of the differentially expressed genes (using the data from placental trophoblasts available from the Roadmap Epigenomics project as background to avoid selection bias (Timmons et al., 2015)) revealed that these genes are largely involved with

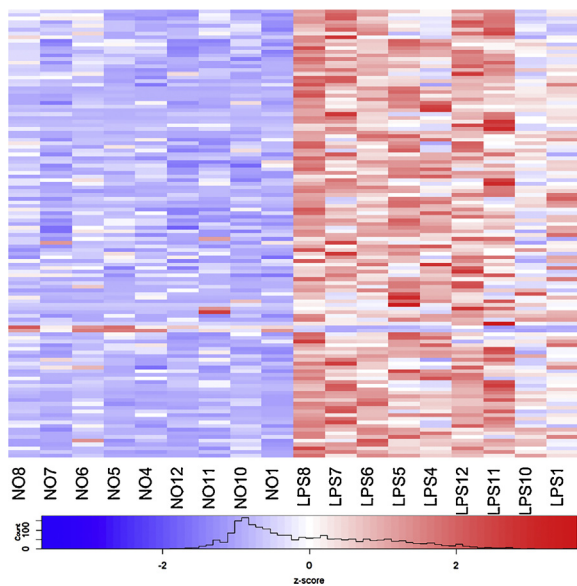


Fig. 1. – Hierarchical cluster analysis of RNAseq data comparing chorionic trophoblasts incubated with conditioned medium from LPS-activated (“LPS”) or resting (“NO”) peripheral blood mononuclear cells from healthy young women. Although there is detectable inter-sample variation, stimulated cells cluster separately from unstimulated cells largely on the basis of genes that show higher expression after stimulation. Rows represent differentially expressed genes for the comparison between LPS and NO and columns indicate individual samples in each group.

leukocyte migration and adhesion as well as the regulation of cytokine production and responses to cytokines (Supplementary Fig. 2).

This finding is consistent with transcriptomic data derived from patients with preterm birth complicated by chorioamnionitis, recently reported by Ackerman et al. (Ackerman et al., 2016). It is interesting to note that, for specific genes, there was considerable inter-sample variation in the intensity of the inflammatory response as reflected in the gene expression data (Fig. 1), a finding consistent with the patient-derived data in the Ackerman paper.

Furthermore, modeling of upstream regulators of the differentially expressed genes using the Ingenuity software platform suggested that these genes are part of a complex Stat1-Stat3 regulatory network (Fig. 2), consistent with our previous findings in primary placental

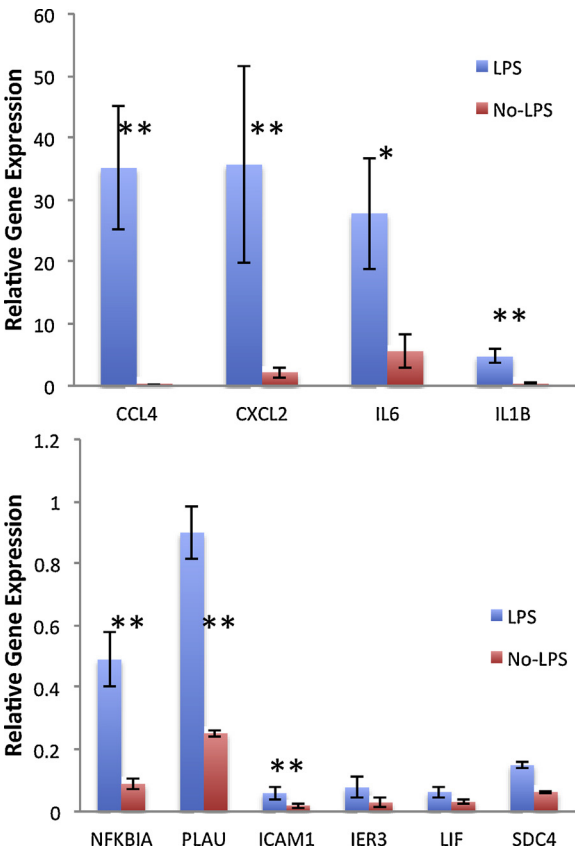


Fig. 3. Validation of the RNA-seq results by real-time quantitative reverse transcription–polymerase chain reaction analysis of 10 genes in an independent cohort (4 LPS samples and 4 control samples). Statistical analysis was performed on ΔCt values using unpaired *t*-tests. Values are the mean \pm SEM. * = $P < 0.05$; ** = $P < 0.01$ versus healthy controls.

trophoblasts (Jiang et al., 2007) as well as the Ackerman data.

3.2. Corroboration of RNAseq data by qRT-PCR

To confirm the differences in gene expression between two groups (LPS samples and control samples) observed in the RNAseq

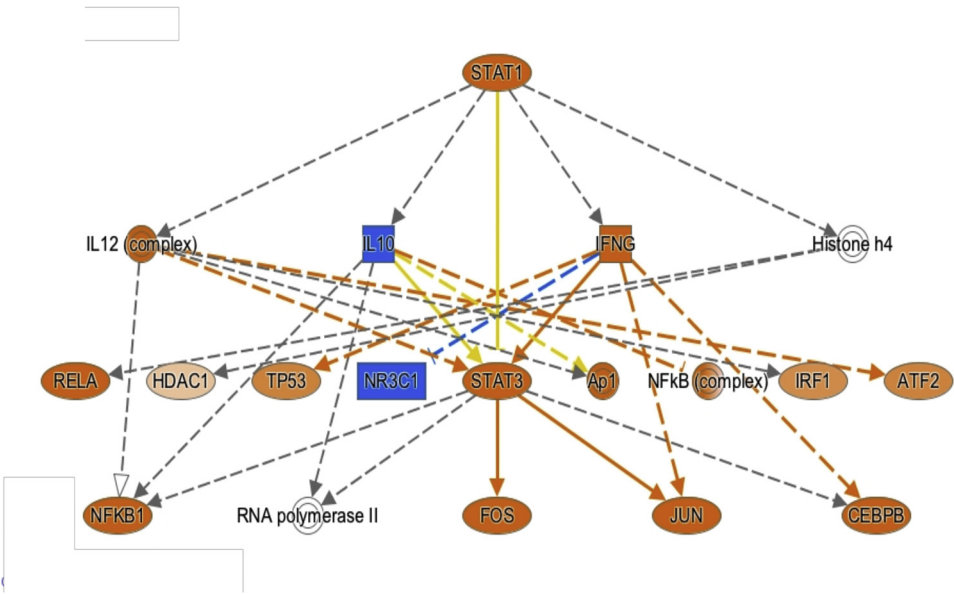


Fig. 2. – Network derived from upstream analysis of RNAseq data comparing unstimulated and stimulated chorionic trophoblasts (CT). Ovals represent transcriptional regulators, circles represent protein complexes (e.g., NFkB) and rectangles represent proteins. The orange colour predicts an activated state based on the patterns of gene expression, and the blue colour represents predicted repression. Note that the predicted activation of STAT1 and STAT3, which we have previously observed directly using this experimental model (Jiang et al., 2007). Note also the predicted activation of the chromatin modifier, histone deacetylase 1 (HDAC1), a finding consistent with our hypothesis that chromatin reorganization is a critical component in the response of CT.

experiments, qRT-PCR was performed in an additional cohort of samples (4 LPS samples and 4 control samples). The results show that 7 of 10 genes differentially expressed between groups in the RNAseq analysis were also differentially expressed in the qRT-PCR (Fig. 3).

3.3. Genome-wide methylation

We found 13,786 methylated sites (500bp bin) in CT. By using CEAS (Shin et al., 2009), we annotated genomic features of methylated regions in trophoblast, yielding similar distributions to the genome background as a whole. The largest portion of methylated regions were observed at introns and distal intergenic sites (Supplementary Fig. 3). This observation corroborated well with other studies that have shown that methylation frequently occurs in intronic as compared to other genomic regions (Hogart et al., 2012; Carrio et al., 2015; Sinclair et al., 2015).

Using bedtools, we searched for the closest transcription start sites (TSS) to the 13,786 methylated regions that we identified via MRE/MeDIP sequencing. We identified 4295 protein coding genes by this process. GOrilla gene ontology (GO) enrichment analysis with those protein coding genes and trophoblast expressed genes as background, identified 13 significant GO processes ($FDR \leq 0.05$, Supplementary Table 5). We identified 181 genes significantly enriched in G-protein coupled receptor signaling pathways. Also, 80 genes were annotated to involve in detection of stimuli ($FDR 0.0004$), both relevant to the response or adaptation to inflammatory mediators.

Next, we sought to identify DMRs and possible functional associations with those regions. We identified 318 DMRs (fold change ≥ 1.5 and $p\text{-value} \leq 0.05$) that were associated with 306 genes (155 protein coding genes). GO enrichment analysis on 155 protein coding genes demonstrated two major groups of significant biological processes ($p\text{-value}$ range from $4.37E-04$ to $8.05E-05$, Supplementary Table 6), although the specific relevance of the identified processes to CT function was obscure. On the whole, stimulated and unstimulated cells showed crisp separation when analyzed by principal components analysis, despite observable inter-sample variation (Fig. 4A and B).

3.4. Correlation between changes in transcription and changes in DNA methylation

We had hypothesized that, under specific circumstances (e.g. a strong enough inflammatory stimulus), genes that are repressed at the maternal-fetal interface via DNA methylation might be de-methylated, allowing either a maternal immune response to the semi-allogenic fetus and/or the onset of early labor. We therefore used the BETA software package to determine whether the observed methylation changes might account for the observed transcriptional changes that occur in CT after incubation with inflammatory mediators. BETA integrates information re: chromatin regulators (in this case, DNA methylation) with differential gene expression data to define the regulatory potential of specific genomic regions (Wang et al., 2013). As shown in Fig. 5, below, the observed methylation changes had negligible impact on the observed transcriptional changes in CT.

Thus, we are unable to conclude that DNA methylation is an important mechanism through which transcriptional control is exerted in CT in response to inflammatory mediators.

4. Discussion

Pregnancy is a time of complex immunologic adaptation, which requires localized and systemic changes to maternal immune responses to prevent immunologic recognition of the semi-allogenic fetus while maintaining the ability to resist invading pathogens. At the same time, the fetal side of the maternal-fetal dyad is also capable of mounting limited immune responses (Jarvis et al., 1995), and we are coming to better understand the deleterious effects of over-exuberant

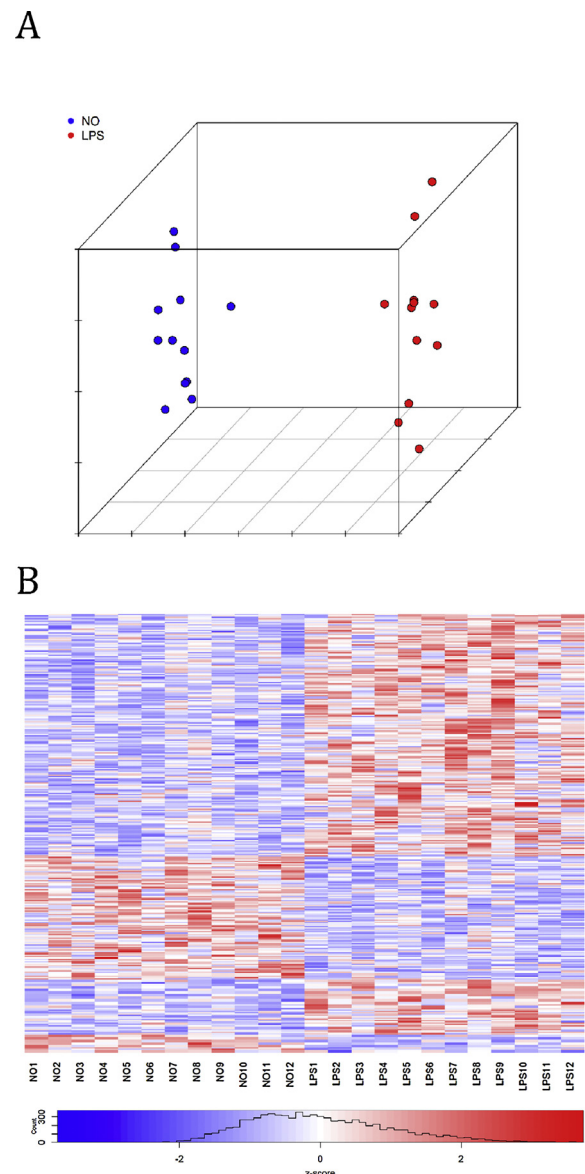


Fig. 4. A – Three dimensional graph showing results from hierarchical cluster analysis of DNA methylation data from chorionic trophoblasts incubated with conditioned medium from LPS-activated (“LPS”) or resting (“NO”) PBMC. Samples from each condition cluster separately. Fig. 4B shows results from hierarchical cluster analysis, demonstrating the degree of inter-sample variation.

inflammatory responses to fetal well-being (Yoon et al., 1997; Kallapur and Jobe, 2006; Edwards and Tan, 2006). Thus, successful pregnancy requires tight control over immune and inflammatory responses from the time of implantation to the start of parturition, and this control must be exerted on both the maternal and fetal side of the maternal-fetal dyad.

In this study, we used an *in vitro* model to better understand the response of fetally-derived CT to inflammatory stimuli generated from PBMC conditioned medium. The results contain a number of interesting findings. The limited number of differentially expressed genes reported here ($n = 114$) is similar to our previous study using JAR chorioncarcinoma cells exposed to PHA-stimulated PBMC conditioned medium (Jarvis et al., 2004). The latter work analyzed gene expression differences to stimuli over time. In that study, we found that 108 genes were differentially expressed after two hours of stimulation, two genes were differentially expressed at 8 h, and 3 genes were differentially

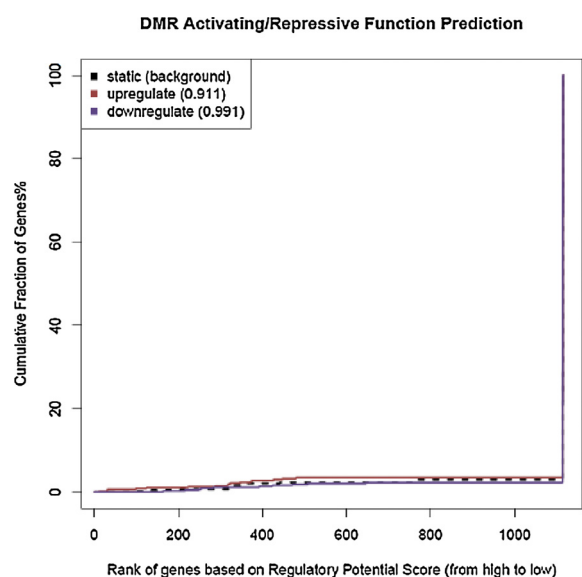


Fig. 5. Results from analysis of the regulatory potential of differentially methylated regions generated using Binding and Expression Target Analysis (BETA) software. By incorporating gene expression and differentially methylated regions, the BETA tool predicted negligible impact on the observed transcription changes seen in the comparison of stimulated and unstimulated chorionic trophoblasts (p-values for up/down regulated genes > 0.01). Axis Y is the proportion of genes that are ranked better than the axis X (regulatory potential score). The red line represents up-regulated genes, the purple line shows down-regulated genes and the dash line indicates background (non-differentially expressed genes). The Kolmogorov-Smirnov test was used to derive p-values (in brackets) to show significance of up/down-regulated genes with respect to non-differentially expressed genes.

expressed after 18 h. These results suggest tight regulation of transcription in trophoblasts and trophoblastic cell lines and raised the question of the underlying mechanisms regulating transcription in trophoblasts within the chorion and placenta.

The genes showing differential expression in CT showed were strongly linked ontologically to pro-inflammatory pathways, as shown in Fig. 2. These findings are consistent with our earlier reports using this same model with primary placental trophoblasts (Jiang et al., 2007). In that paper, we demonstrated that mitogen-activated protein kinase pathways are activated and both STAT1 and STAT3 are phosphorylated within minutes of cell stimulation. Phosphorylated STAT1 and STAT3 can be detected within the nuclei of cultured trophoblasts within 15 min of stimulation.

One of the interesting findings from this study was the degree of inter-sample variation that we were able to detect, especially in the RNAseq data. While these samples were stimulated with conditioned medium generated from PBMC of 2 different donors, hierarchical cluster analysis did not impute differences between the 2 donors as a source of inter-sample variability. These data reflect, instead, the considerable degree of variability that one encounters with human samples, and faithfully recapitulate, in that respect, the data from the Ackerman study (Ackerman et al., 2016). Furthermore, recently published data have demonstrated that these inter-patient variations may be critical determinants of fetal outcome (Pappas et al., 2015). We propose that these inter-individual variations are likely to have an underlying genetic/epigenetic basis and may provide the underlying basis for genetic risk for preterm labor in the context of specific environmental challenges, including infections.

In this paper, we exclude one potential epigenetic regulator mediating transcriptional changes in CT: DNA methylation. We found only modest differences in the methylation profiles of CT before and after stimulation. Furthermore, computational analysis of the differentially methylated regions failed to demonstrate a regulatory relationship

between those regions and differentially expressed genes. For some critical genes, in particular HLA class I and class II genes, there was no association between methylation status (the promoters of all these genes were unmethylated) and expression before or after stimulation (failure of CT to express HLA even after an inflammatory stimulus is well-documented). It is reasonable, therefore, to hypothesize that both expression of inflammatory mediators as observed in RNAseq experiments and suppression of HLA class I and II is mediated by other epigenetic factors, which may include CTCF-mediated chromatin re-organization (Hnisz et al., 2016), specific histone marks, etc. In support of this idea is the predicted activation of histone deacetylase 1 (HDAC1) as shown in Fig. 2. Histone acetylation removes a positive charge on the N terminus of the histone tails that normally interacts with negatively charged phosphates on the DNA backbone. Acetylation thus leads to a more open chromatin structure and transcriptional activation. HDAC reverses histone acetylation, leading to a more condensed chromatin structure (Giles et al., 2010). There are direct, experimental approaches that can test this hypothesis, e.g., using the assay for transposase-accessible chromatin-sequencing (Buenrostro et al., 2015).

There are several limitations that need to be considered in interpreting our data. For example, it is possible that, had we incubated CT for time periods longer than 2 h, we might have seen more extensive methylation changes. However, that possibility does not alter the current findings, i.e., that the transcriptional changes observed after 2 h could not be attributed to methylation changes. We also note that the PBMC used to produce conditioned medium were obtained from 2 healthy, non-pregnant women. While this approach allows us to compare data with our previous studies, it is possible that conditioned medium prepared from PBMC of pregnant women would have produced different levels of the most potent inflammatory stimuli, given the broad immunosuppression observed in human pregnancy. At the same time, this would not change the essential findings, which were directed at addressing the question, “How to CT respond to inflammatory stimuli, and by what mechanism do they re-organize their transcriptomes?”

In conclusion, we find that CT display complex patterns of transcription in response to inflammation. DNA methylation does not appear to be an important regulator of the observed transcriptional changes, inviting focused inquiries into the identification of those epigenetic mechanisms that are most relevant.

Conflicts of interest

The authors affirm that they have no conflicts of interest, including any financial, personal or other relationships with other people or organizations, nor have any such relationships existed within three years of beginning this work. The authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi: <https://doi.org/10.1016/j.jri.2018.05.005>

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